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ORIGINAL ARTICLE

Calcineurin inhibitor-free GVHD prophylaxis with sirolimus, mycophenolate mofetil and ATG in Allo-SCT for leukemia patients with high relapse risk: an observational cohort study

M Schleuning, D Judith, Z Jedlickova, T Stübig, M Heshmat, H Baurmann and R Schwerdtfeger

Centre for Bone Marrow and Blood Stem Cell Transplantation, Deutsche Klinik für Diagnostik, Wiesbaden, Germany

Certain leukemias have a high relapse risk even after allo-SCT, and GVHD prophylaxis with calcineurin inhibitors (CNIs) may interfere with a possible GVL effect. Therefore, we replaced CYA by sirolimus in patients with high relapse risk. In contrast to CNIs, sirolimus promotes the generation of regulatory T-cells and has potent antineoplastic activity. Sirolimus has been used in combination with CNI for GVHD prophylaxis in hematopoietic SCT. However, no CNI-free prophylactic regimen with sirolimus has been evaluated so far. Within the FLAMSA-RIC protocol, 15 patients received GVHD prophylaxis with sirolimus and mycophenolate mofetil (MMF). The underlying diagnoses were relapsed or refractory T-ALL ($n=3$), AML with FMS-like tyrosine kinase 3-internal tandem duplication (FLT3-ITD) or mixed-lineage leukemia-partial tandem duplication (MLL-PTD; $n=10$; 5 with refractory disease) and CML in refractory myeloid blast crisis ($n=2$). All evaluable patients ($n=14$) were engrafted. Grades II–IV acute GVHD occurred in 21% and chronic GVHD in 30% of patients. Non-relapse mortality rate was 14%. No thrombotic microangiopathy or sinusoidal obstruction syndrome was observed. Three patients with FLT3-ITD+ AML relapsed after a median of 112 days. At a median follow-up of 10 months after transplantation, 10 patients are alive and in complete remission. In conclusion, sirolimus-based GVHD prophylactic regimens deserve further investigation.

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Introduction

Recently, we have developed a sequential regimen of chemotherapy and reduced-intensity conditioning for allo-SCT for patients with high-risk acute leukemia, which has become known as the FLAMSA-RIC protocol.[1] Also this protocol seems to be rather effective even in refractory leukemia,[2] early relapses remain a serious challenge in very high-risk leukemia. Besides the biological nature of these very high-risk leukemias, immunosuppression after allografting with calcineurin inhibitors (CNIs) might contribute to an increased risk of relapse by interfering with a possible GVL effect. Therefore, immunosuppressive drugs with the ability to ensure engraftment and to prevent severe GVHD, which have some antileukemic efficacy in parallel, might have the potency to bridge the time of necessary immunosuppression until a GVL effect can be exploited.

Sirolimus exerts its action by binding to FK-binding protein 12 (FKBP12) and subsequently forming a complex with the mammalian target of rapamycin (mTOR) and the raptor/rictor proteins.[3,4] The generation of this complex results in cell cycle arrest in G1 through the inhibition of DNA transcription, DNA translation and protein synthesis. In contrast to CNI, sirolimus promotes the generation of CD4+CD25+FoxP3+ regulatory T-cells. As this process is IL-2-dependent, it is blocked by CNIs, but seems to be independent of inosine monophosphate dehydrogenase inhibition by mycophenolate mofetil (MMF).[5] Clinically, sirolimus has been proven to be effective as prophylaxis of allograft rejection in solid organ transplantation,[6,7] and in combination with CNI for GVHD prophylaxis after related and unrelated hematopoietic SCT,[8] as well as for therapy of acute[9] and chronic GVHD.[10–12]

In addition to its immunosuppressive effects, sirolimus also has a potent antineoplastic activity. Antitumoral activity of sirolimus and its analogs temsirolimus or everolimus (CCI-779, RAD001) has been studied in various preclinical solid tumor models, and appears very efficient on tumors displaying activation of the phosphoinositide-3 kinase/AKT pathway.[13–15] Phase I/II clinical trials are in progress with this class of substances in various solid cancers[16] and in hematological malignancies.[17,18] In the evolution of AML, constitutive activation of

Correspondence: Professor Dr M Schleuning, Centre for Bone Marrow and Blood Stem Cell Transplantation, Stiftung Deutsche Klinik für Diagnostik, Aukammallee 33, Wiesbaden 65191, Germany.
E-mail: schleuning.kmt@dkd-wiesbaden.de
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the phosphoinositide-3 kinase pathway is a critical event.[19] One of the important downstream targets of phosphoinositide-3 kinase is mTOR, which mediates the effects of both BCR-ABL and FMS-like tyrosine kinase 3 (FLT3) by the regulation of protein translation through phosphorylation of its substrates, p70 S6 kinase and 4EBP-1.[20] Inhibition of mTOR with rapamycin inhibits proliferation of cells from patients with AML and FLT3 mutations, and in patients with relapsed/refractory or poor-risk AML, sirolimus has been reported to induce significant clinical responses as a single agent.[21] Similarly, the mTOR pathway seems to play a critical role in the pathogenesis of T-ALL, where it is deregulated as a downstream target by constitutive NOTCH activation, and T-ALL cell growth was suppressed in a highly synergistic manner by simultaneous treatment with the mTOR inhibitor sirolimus and NOTCH blockade by γ-secretase inhibitors.[22]

The most important adverse reactions attributable to sirolimus are reversible cytopenias,[23] interstitial pneumonitis,[24] cutaneous reactions and mucosal ulcers,[25,26] hyperlipidemia[27] and impaired wound healing.[28] In contrast to CNI, sirolimus is neither neurotoxic nor nephrotoxic. However, in combination with CNI, an increased incidence of transplantation-associated thrombotic microangiopathy and possibly sinusoidal obstruction syndrome has been observed.[8,29] On the other hand, sirolimus exerts protection against viral infections, especially CMV reactivation.[30]

On the basis of the observations mentioned, we changed the GVHD prophylaxis within the FLAMSA-RIC protocol from CYA and MMF to sirolimus, and MMF for leukemia patients with high relapse risk. This paper summarizes the results of this strategy in a first cohort of 15 consecutive patients.

Patients and methods

Study design

Within the FLAMSA-RIC conditioning protocol for allogeneic PBSC transplantation, the GVHD prophylaxis was changed from CYA/MMF to sirolimus/MMF in June 2006 in patients with high relapse risk. High relapse risk was assumed in patients with primary refractory or relapsed T-ALL after allogeneic transplantation, in patients with tyrosine kinase inhibitor-resistant CML in blast crisis and in patients with AML with Flt3 or mixed-lineage leukemia mutations, regardless of the remission status. The protocol has been approved by the local ethical review board and all patients have signed an informed consent. Data were analyzed retrospectively as of 31 March 2008 after a cohort of 15 patients had received this GVHD prophylaxis. The analysis included the incidence of graft failure, the incidence and the severity of acute and chronic GVHD, treatment-related mortality, relapse rate and the incidence of serious adverse events related to the combination of sirolimus and MMF.

Preparative regimen

The preparative regimen was the FLAMSA-RIC protocol, essentially as published.[1] Shortly, after a 4-day chemotherapy cycle consisting of Amsacrine at a dose of 100 mg/m^2, fludarabin 30 mg/m^2 and cytosine-arabinoside 2000 mg/m^2 and 3 days rest, patients received TBI at a single dose of 400 cGy, followed by CY (40 or 60 mg per kg body weight in case of an unrelated donor) on two consecutive days. Patients with myeloid malignancies also received ATG during conditioning (ATG-Fresenius at a total dose of 15 mg per kg body weight or 45 mg/kg in case of unrelated donors given in equal doses from day −3 to day −1). One patient with an unrelated donor received thymoglobulin (total dose 7.5 mg/kg) instead of ATG-Fresenius. Sirolimus was administered orally from day −1 in a dose of 2 mg twice daily. Serum concentrations of sirolimus were monitored thereafter twice a week with target concentrations between 5 and 10 ng/ml during hospitalization and then as clinically indicated. In the absence of GVHD, sirolimus should have been tapered between days +60 and +90. The first dose (1000 mg) of MMF was administered intravenously 6–12 h after transplantation and thereafter 2000 mg were administered in two daily doses. After engraftment, oral formulations of MMF were applied. In the absence of GVHD, the dosage was reduced and terminated at day +50, if possible. No CNI or MTX was given after transplantation. Growth factor-mobilized PBSCs were used in all but one patient as a stem cell source. In both patients with prior allogeneic transplantation, a second HLA-identical sibling donated PBSC. Beginning from day +120, patients free of GVHD and of immunosuppression for at least 30 days were eligible for adjuvant treatment with escalating doses of donor lymphocyte infusions.

All patients were treated in single-patient rooms with positive pressure high efficiency particulate air (HEPA)-filtered air at least until engraftment, and routine laboratory tests were performed on a daily basis. Chimerism analysis was performed regularly using short tandem repeat polymerase chain reaction (STR-PCR). Patients received prophylactic antiviral therapy against herpes virus infections and after engraftment prophylaxis against *Pneumocystis carinii*. All patients were monitored for CMV reactivation. No prophylactic treatment against CMV was given. Acute and chronic GVHD were graded according to the Gluckman and Shulman criteria.[31,32]

Statistical analysis

Neutrophil engraftment was defined as the first of three consecutive days of an absolute neutrophil count of 500 cells/μl and plt engraftment as the first day of an unsupported plt count of 20 000/μl. Leukemia-free survival was defined as the time from transplantation to relapse or death from any cause. Overall survival was defined as the time from transplantation to death from any cause. Patients alive without relapse were censored at the date of last contact. Descriptive statistics were used when applicable, and for comparative analyses, the Fisher exact test was used. Leukemia-free survival and overall survival were calculated according to the method of Kaplan and Meier.[33] With respect to acute GVHD, the cumulative incidence was calculated with 100-day death as a competing risk. All analyses were performed with the SPSS software

Table 1 Patient characteristics

UPN	*Patient (age/sex)*	*Donor (type/sex)*	*Disease status at transplantation*	*Karyotype and molecular genetics*	*HCT-CI*	*CMV-IgG (receptor/donor)*
701	26/M	Sib-id/F	T-ALL; relapse after allo-transplant	46XY	3	pos/.pos.
724	38/M	Sib-id/F	T-ALL; primary induction failure; refractory to nelarabine and forodesine	Complex	1	pos./pos.
731	33/M	Sib-id/F	AML-M5a; early relapse	46XY; Flt3-ITD pos.	4	pos/.pos.
739	61/F	URD-id/F	CML-my.BC imatinib/chemotherapy resistant	t(9;22), inv(16)	1	pos./pos.
747	62/F	URD-id/F	AML-M2; primary induction failure	46XX; Flt3-ITD pos.	4	pos./pos.
749	39/F	URD-id/M	AML-M4; refractory relapse after auto-transplant	46XX; Flt3-ITD, NPM1 pos.	3	neg./pos.
757	52/F	URD-df/M (HLA-C-MM)	AML-M2; CR1	46XX; Flt3-ITD, MLL-PTD pos.	2	pos./neg.
780	44/F	URD-df/M (HLA-B-MM)	AML-M4; refractory relapse	46XX; Flt3-ITD, NPM1 pos.	2	pos./neg.
786	40/F	Sib-id/M	AML-M5b; CR1	46XX; Flt3-ITD, NPM1 pos.	2	neg./neg.
788	24/F	URD-df/F HLA-A-MM)	AML-M1/2; primary induction failure	46XX; Flt3-ITD, MLL-PTD pos.	2	neg./pos.
793	52/F	URD-id/M	AML-M0; CR1	Complex; MLL-PTD pos.	1	pos./pos.
798	50/F	URD-id/F	AML-M2; CR1	46XX; Flt3-ITD, NPM1 pos.	0	pos./pos.
808	53/F	Sib-id/F	AML-M1; CR1	46XX; Flt3-ITD pos.	1	pos./pos.
812	59/M	URD-id/M	CML-my.BC Imatinib/dasatinib/chemotherapy resistant	t(9;22), complex T351I-mutation	0	neg./neg.
849	49/M	Sib-id/F	T-ALL; relapse after allo-transplant, nelarabine resistant	Complex	3	neg./neg.

Abbreviations: F = female; HCT-CI = Hematopoietic cell transplantation-specific comorbidity index; M = male; neg. = negative; pos. = positive; Sib-id = HLA-identical sibling; URD-df = HLA mismatched donor (the type of mismatch (MM) is indicated in the line below; URD-id = fully HLA matched unrelated donor.

package (SPSS Inc, Chicago, IL, USA) and all *P*-values are two-sided.

Results

Patient and transplant characteristics

Fifteen patients (median age 49 years; range 24–62) transplanted between June 2006 and October 2007 received GVHD prophylaxis with sirolimus and MMF. Patient characteristics including comorbidity[34] are summarized in Table 1. The first two patients received grafts from an HLA-identical sibling donor. After these patients had stable engraftment, patients with HLA matched, unrelated donors were recruited. Finally, six patients were grafted from a sibling and nine from a high-resolution HLA matched (A, B, C, DRB1 and DQB1), unrelated donor. All but one patient received growth factor mobilized-PBSCs at a median dose of 7.8×10^6 (range: $2.9–17.0 \times 10^6$) $CD34^+$ cells per kg body weight of the recipient as graft. One patient received BM with 3.73×10^8 mononucleated cells per kg body weight, because the registry refused to collect growth factor-mobilized PBSCs.

Engraftment

All but one patient who died on day +6 from invasive aspergillosis had neutrophil engraftment after a median of 20 days (range: 14–32) without the use of hematopoiesis-stimulating factors. No significant correlation to the graft cell dose, to the disease status at transplantation, to the type of donor, to the presence of a HLA mismatch or to medium serum trough levels of sirolimus could be found, as analyzed by Fisher's exact test. One heavily pretreated patient never achieved stable plt engraftment of more than 20 000/μl during the observation period until day +123. All other patients had plt engraftment after a median of 16 days (range: 11–31) and more than 100 000 plts/μl after a median of 29 days (range: 14–147). By day +30, all evaluable patients were complete chimera in peripheral blood and all survived to first hospital discharge (median day +36; range: days +30 to +75).

Acute GVHD

Only two patients developed grades II–IV acute GVHD and three more patients had a rash of less than 25% of the body surface, which resolved in two patients with topical steroids only. Both patients with grades II–IV acute GVHD required systemic steroid therapy. In the patient with grade II acute GVHD, it was limited to the skin and responsive to steroids. The patient with grade IV acute GVHD developed it when already being outpatient on day +36 and delayed hospital admission to day +41, despite severe diarrhea and skin rash. There was no response to systemic steroids; therefore, the patient was additionally treated with tacrolimus, MoAbs directed against tumor necrosis factor-α (TNF-α) and IL-2 receptor and extracorporal photopheresis. Despite these efforts, the patient died because of acute GVHD on day +71. In a competing risk model including death from any cause up to day +100, the relative risk of developing grades II–IV acute GVHD was 22.5% (Figure 1).

Chronic GVHD

De novo chronic GVHD developed in 4 (30%) out of 13 patients surviving past day +100 after tapering sirolimus. In three patients with the involvement of the skin and oral

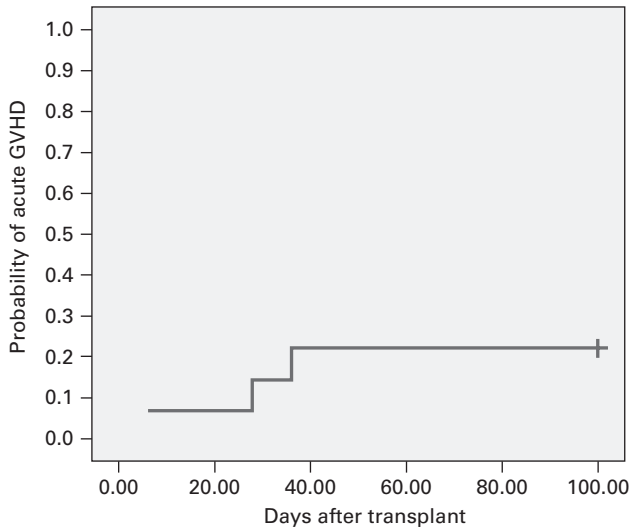

Figure 1 Cumulative incidence of acute GVHD with death as a competing risk. The cumulative incidence of grades II–IV acute GVHD was 22.5%.

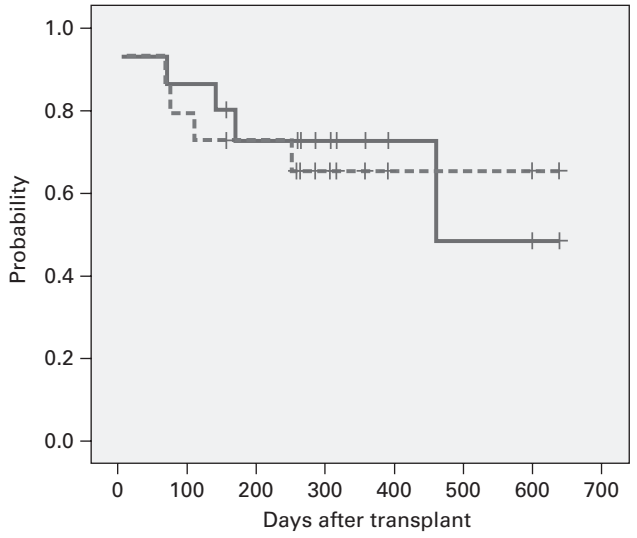

Figure 2 Kaplan–Meier estimates of overall (solid line) and leukemia-free survival (dotted line).

mucosa, it became extensive. In all three patients, the dose of sirolimus was then increased again to therapeutical doses and two required an additional short course of steroids to relieve their symptoms. One patient developed limited chronic GVHD of the skin not requiring any medical intervention after sirolimus was terminated.

Sirolimus/MMF attributable toxicity

The assessment of safety focused on identifying toxicities associated with the combination treatment of sirolimus and MMF. As noted, neutrophil engraftment was somewhat delayed until day +20. One patient suffered from HHV6-associated erosive gastritis, which necessitated laser coagulation. After terminating sirolimus earlier than planned already at day +48, the patient's condition improved. In another patient, sirolimus was replaced by everolimus because of suspected pneumonitis,[35] which, however, turned out to be *Pneumocystis jirovecii* pneumonia after bronchoalveolar lavage was performed and it responded well to high-dose trimethoprim/sulfamethoxazole treatment. All other patients remained on sirolimus until the planed termination of immunosuppression or until relapse. At the time of analysis, three patients were still under treatment with sirolimus. Hyperlipidemia was a common phenomenon with hypertriglyceridemia occurring in 14/15 patients with a median maximum level of 438.5 mg/100 ml (range: 278–1112) and mild hypercholesterolemia occurring in 10/15 patients with a median maximum level of 244.5 mg/100 ml (range: 203–445). There was no direct correlation between hyperlipidemia and sirolimus serum trough levels, and it resolved spontaneously in all cases after sirolimus has been tapered. No specific treatment was administered. No renal impairment, no transplant associated microangiopathy, no hemolytic uremic syndrome or sinusoidal obstruction syndrome was observed.

Table 2 Causes of death

	Combined, no. (%)	*MRD, no. (%)*	*URD, no. (%)*
Relapse	3 (20.0)	1 (16.7)	2 (22.2)
GVHD	1 (6.7)	0	1 (11.1)
Infection	1 (6.7)	0	1 (11.1)

Abbreviations: MRD = matched related donor; URD = unrelated donor.

CMV reactivation

Although 10/15 recipients were CMV seropositive and no CMV-specific prophylaxis was used, we did not observe any systemic CMV reactivation during the observation period.

Survival and relapse incidence

After a median follow-up of 10 months after transplantation for surviving patients, the probability of LFS and OS at 1 year is 67 and 72%, respectively (Figure 2). The non-relapse mortality rate was only 14% during the whole observation period. Three patients with FLT3-ITD relapsed after a median of 112 days. Two of those had been transplanted in refractory disease and one in CR1 and one patient relapsed despite having extensive chronic GVHD at day +225, primarily at extramedullary sites (Table 2). In one of the relapsed patients, sirolimus was terminated earlier than planned on day +48 because of HHV6-associated erosive gastritis. Of 10 patients, 5 surviving past day +120 received adjuvant donor lymphocyte infusions (aDLI) starting at day +157 (median, range: days +110 to +294) in escalating doses after terminating immunosuppression after a median of 90 days (range 60–159). Out of the scheduled three infusions, one patient received only two infusions, because of developing mild oral lichenoid chronic GVHD. The applied CD3+ lymphocyte doses were 1×10^6, 1×10^7 and 5×10^7 per kg body weight of the host with sibling donors and 2×10^5, 2×10^6 and 1×10^7 per kg body weight of the host in case of unrelated donors,

respectively. None of these patients relapsed during the observation period. All patients were complete chimera in peripheral blood before receiving the first aDLI. Reasons for not giving aDLI in the remaining five patients were either active GVHD or ongoing immunosuppression. One patient developed acute and then chronic GVHD after the third aDLI.

Discussion

This study clearly shows that a CNI-free GVHD prophylaxis with sirolimus and MMF in matched related and unrelated allo-SCT is feasible as no primary graft failure occurred. However, time to neutrophil engraftment was 6 days longer, as it has been reported with the same regimen and a CYA-based GVHD prophylaxis.[1,2] No significant contributing factor could be identified by Fisher's exact test, but it should be stressed that no granulopoiesis-stimulating agents were used. By contrast, no prolonged period of thrombocytopenia was observed. As reported by other groups, there was no delayed neutrophil engraftment if sirolimus was used in combination with CNIs.[8,36] However, the study by the Boston group also used G-CSF, if necessary. If the GVHD prophylaxis with the combination of sirolimus and MMF is indeed the reason for the observed prolonged neutropenia remains to be proven. Both drugs are, however, potentially myelotoxic and some sort of synergism between both drugs has been discussed in this respect.[23,37–39]

The relative risk for developing grades II–IV acute GVHD was 22.5%, and thus acute GVHD seems to be well controlled with this prophylaxis. Although the duration of prophylactic immunosuppression was rather short, and our patient cohort was 5-year older, and all suffered from very high-risk disease, the incidence of acute GVHD is comparable with that reported by the Boston group with the combination of tacrolimus and sirolimus[8] and much lower than the more than 70% incidence reported by the Seattle group, who used the combination of sirolimus, CNI and short-course MTX.[36] However, both groups did not incorporate ATG into their preparative regimen, which might be one reason for a higher incidence of acute GVHD. It was also lower than in the earlier reported FLAMSA studies utilizing CYA, MMF and ATG as GVHD prophylaxis, and the reported incidence of grades II–IV acute GVHD was 49 and 28% without correction for the competing risk factor death.[1,2] Possibly by enhancing the numbers and function of CD4+CD25+ regulatory T cells,[5] the combination of sirolimus and MMF seems to be a very efficient strategy for prophylaxis of acute GVHD and is at least as effective as CNI-based protocols. The 30% incidence of chronic GVHD is comparable with what we have seen in earlier studies[1,2] utilizing the FLAMSA preparative regimen (32–45%), but is much lower than in those studies (59–90%) that combined CNI and sirolimus.[8,36] As all three patients, who did not receive ATG during the preparative regimen, developed chronic GVHD and there is no difference to the earlier FLAMSA studies, the low incidence of chronic GVHD might be partly explained by the use of ATG.[40]

In general, toxicities associated with the combination treatment with sirolimus and MMF were moderate. However, in conditions requiring effective wound healing, sirolimus should be used with caution because it inhibits PDGF and basic fibroblast growth factor,[41] thus possibly interfering with wound healing.[28] In addition, mucosal ulcers are common adverse events of sirolimus.[25,26] Reversible hyperlipidemias were frequent but never required therapeutic intervention. There was no renal impairment, no transplant-associated microangiopathy, no hemolytic uremic syndrome or sinusoidal obstruction syndrome, conditions which have frequently been reported when sirolimus was used in combination with CNIs.[8,36]

Of interest, we did not observe any systemic CMV reactivation, despite the seropositivity of two of three of the patients. This is in accordance with observations suggesting a CMV protective effect of sirolimus-based immunosuppression after solid organ transplantation[42,43] and, as recently published, also after hematopoietic SCT.[30] The mechanism(s) by which sirolimus may exert this protective effect remain to be determined.

The non-relapse mortality rate of 14% during the whole observation period reflects the excellent tolerability of this GVHD prophylaxis, and a relapse incidence of 23% seems rather low in this cohort of the highest risk leukemia patients, and of special interest none of the T-ALL patients relapsed so far. In addition, on account of the low incidence of acute GVHD, this prophylactic regimen also provides an excellent platform for adjuvant immunotherapy, as 50% of the patients surviving past day +120 could receive aDLI as compared with 24% in the earlier FLAMSA studies.[1]

Although results are preliminary because of short follow-up and low patient numbers, this GVHD prophylaxis seems to offer a promising option to transplant very high-risk leukemia patients, even with diseases so far believed to be incurable, such as primarily refractory or relapsed T-ALL after prior allo-transplantation. Its antileukemic effects in FLT3-ITD+ or mixed-lineage leukemia rearranged AML deserve further investigation.

In summary, GVHD prophylaxis with sirolimus and MMF is feasible and should further be evaluated in larger prospective trials.

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References

1 Schmid C, Schleuning M, Ledderose G, Tischer J, Kolb HJ. Sequential regimen of chemotherapy, reduced-intensity conditioning for allogeneic stem-cell transplantation, and prophylactic donor lymphocyte transfusion in high-risk acute myeloid leukemia and myelodysplastic syndrome. *J Clin Oncol* 2005; **23**: 5675–5687.
2 Schmid C, Schleuning M, Schwerdtfeger R, Hertenstein B, Mischak-Weissinger E, Bunjes D *et al*. Long-term survival in refractory acute myeloid leukemia after sequential treatment with chemotherapy and reduced-intensity conditioning

for allogeneic stem cell transplantation. *Blood* 2006; **108**: 1092–1099.
3 Ali SM, Sabatini DM. Structure of S6 kinase 1 determines whether raptor-mTOR or rictor-mTOR phosphorylates its hydrophobic motif site. *J Biol Chem* 2005; **280**: 19445–19448.
4 Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H *et al*. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 2004; **14**: 1296–1302.
5 Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J *et al*. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 2006; **108**: 390–399.
6 Johnson RW. Sirolimus (Rapamune) in renal transplantation. *Curr Opin Nephrol Hypertens* 2002; **11**: 603–607.
7 Trotter JF. Sirolimus in liver transplantation. *Transplant Proc* 2003; **35**: S193–S200.
8 Cutler C, Li S, Ho VT, Koreth J, Alyea E, Soiffer RJ *et al*. Extended follow-up of methotrexate-free immunosuppression using sirolimus and tacrolimus in related and unrelated donor peripheral blood stem cell transplantation. *Blood* 2007; **109**: 3108–3114.
9 Benito AI, Furlong T, Martin PJ, Anasetti C, Appelbaum FR, Doney K *et al*. Sirolimus (rapamycin) for the treatment of steroid-refractory acute graft-versus-host disease. *Transplantation* 2001; **72**: 1924–1929.
10 Johnston LJ, Brown J, Shizuru JA, Stockerl-Goldstein KE, Stuart MJ, Blume KG *et al*. Rapamycin (sirolimus) for treatment of chronic graft-versus-host disease. *Biol Blood Marrow Transplant* 2005; **11**: 47–55.
11 Couriel DR, Saliba R, Escalon MP, Hsu Y, Ghosh S, Ippoliti C *et al*. Sirolimus in combination with tacrolimus and corticosteroids for the treatment of resistant chronic graft-versus-host disease. *Br J Haematol* 2005; **130**: 409–417.
12 Jurado M, Vallejo C, Pérez-Simón JA, Brunet S, Ferra C, Balsalobre P *et al*. Sirolimus as part of immunosuppressive therapy for refractory chronic graft-versus-host disease. *Biol Blood Marrow Transplant* 2007; **13**: 701–706.
13 Hidalgo M, Rowinsky EK. The rapamycin-sensitive transduction pathway as a target for cancer therapy. *Oncogene* 2000; **19**: 6680–6686.
14 Neshat MS, Mellinghoff IK, Tran C, Stiles B, Thomas G, Petersen R *et al*. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc Natl Acad Sci USA* 2001; **98**: 10314–10319.
15 Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J *et al*. An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten−/− mice. *Proc Natl Acad Sci USA* 2001; **98**: 10320–10325.
16 Raymond E, Alexandre J, Faivre S, Vera K, Materman E, Boni J *et al*. Safety and pharmacokinetics of escalated doses of weekly intravenous infusion of CCI-779, a novel mTOR inhibitor, in patients with cancer. *J Clin Oncol* 2004; **22**: 2336–2347.
17 Platzbecker U, Haase M, Herbst R, Hänel A, Voigtmann K, Thiede CH *et al*. Activity of sirolimus in patients with myelodysplastic syndrome—results of a pilot study. *Br J Haematol* 2005; **128**: 625–630.
18 Haritunians T, Mori A, O'Kelly J, Luong QT, Giles FJ, Koeffler HP. Antiproliferative activity of RAD001 (everolimus) as a single agent and combined with other agents in mantle cell lymphoma. *Leukemia* 2007; **21**: 333–339.
19 Xu Q, Simpson S-E, Scialla TJ, Bagg A, Carroll M. Survival of acute myeloid leukemia cells requires PI3 kinase activation. *Blood* 2003; **102**: 972–980.
20 Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by RAP/mTOR. *Genes Dev* 2001; **15**: 807–826.
21 Recher C, Beyne-Rauzy O, Demur C, Chicanne G, Dos Santos C, Mansat-De Mas V *et al*. Antileukemic activity of rapamycin in acute myeloid leukemia. *Blood* 2005; **105**: 2527–2534.
22 Chan SM, Weng AP, Tibshirani R, Aster JC, Utz PJ. Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukaemia. *Blood* 2007; **110**: 278–286.
23 Hong JC, Kahan BD. Sirolimus-induced thrombocytopenia and leukopenia in renal transplant recipients: risk factors, incidence, progression, and management. *Transplantation* 2000; **69**: 2085–2090.
24 Champion L, Stern M, Israël-Biet D, Mamzer-Bruneel MF, Peraldi MN, Kreis H *et al*. Brief communication: sirolimus-associated pneumonitis: 24 cases in renal transplant recipients. *Ann Intern Med* 2006; **144**: 505–509.
25 Mahé E, Morelon E, Lechaton S, Sang KH, Mansouri R, Ducasse MF *et al*. Cutaneous adverse events in renal transplant recipients receiving sirolimus-based therapy. *Transplantation* 2005; **97**: 476–482.
26 Smith MP, Newstead CG, Ahmad N, Lewington AJ, Tibble S, Lodge JP *et al*. Poor tolerance of sirolimus in a steroid avoidance regimen for renal transplantation. *Transplantation* 2008; **85**: 636–639.
27 Tenderich G, Fuchs U, Zittermann A, Muckelbauer R, Berthold HK, Koerfer R. Comparison of sirolimus and everolimus in their effects on blood lipid profiles and haematological parameters in heart transplant recipients. *Clin Transplant* 2007; **21**: 536–543.
28 Kuppahally S, Al-Khaldi A, Weisshaar D, Valantine HA, Oyer P, Robbins RC *et al*. Wound healing complications with *de novo* sirolimus versus mycophenolate mofetil-based regimen in cardiac transplant recipients. *Am J Transplant* 2006; **6**: 986–992.
29 Cutler C, Henry NL, Magee C, Li S, Kim HT, Alyea E *et al*. Sirolimus and thrombotic microangiopathy after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**: 551–557.
30 Marty FM, Bryar J, Browne SK, Schwarzberg T, Ho VT, Bassett IV *et al*. Sirolimus-based graft-versus-host disease prophylaxis protects against cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation: a cohort analysis. *Blood* 2007; **110**: 490–500.
31 Glucksberg H, Storb R, Fever A, Buckner CD, Neiman PE, Clift RA *et al*. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. *Transplantation* 1974; **18**: 295–304.
32 Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE *et al*. Chronic graft-versus-host syndrome in man. A long term clinicopathologic study of 20 Seattle patients. *Am J Med* 1980; **69**: 204–217.
33 Kaplan E, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958; **53**: 457–481.
34 Sorror ML, Maris MB, Storb R, Baron F, Sandmaier BM, Maloney DG *et al*. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. *Blood* 2005; **106**: 2912–2919.
35 De Simone P, Petruccelli S, Precisi A, Carrai P, Doria R, Menichetti F *et al*. Switch to everolimus for sirolimus-induced pneumonitis in a liver transplant recipient—not all proliferation signal inhibitors are the same: a case report. *Transplant Proc* 2007; **39**: 3500–3501.
36 Furlong T, Kiem HP, Appelbaum FR, Carpenter PA, Deeg HJ, Doney K *et al*. Sirolimus in combination with cyclosporine or tacrolimus plus methotrexate for prevention of graft-versus-host disease following hematopoietic cell transplantation from

unrelated donors. *Biol Blood Marrow Transplant* 2008; **14**: 531–537.
37 Nogueras F, Espinosa MD, Mansilla A, Torres JT, Cabrera MA, Martín-Vivaldi R. Mycophenolate mofetil-induced neutropenia in liver transplantation. *Transplant Proc* 2005; **37**: 1509–1511.
38 Kreis H, Cisterne JM, Land W, Wramner L, Squifflet JP, Abramowicz D *et al*. Sirolimus in association with mycophenolate mofetil induction for the prevention of acute graft rejection in renal allograft recipients. *Transplantation* 2000; **69**: 1252–1260.
39 Busca A, Locatelli F, Moscato D, Falda M. Sirolimus-related toxicity in stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**: 647–649.
40 Basara N, Baurmann H, Kolbe K, Yaman A, Labopin M, Burchardt A *et al*. Antithymocyte globulin for the prevention of graft-versus-host disease after unrelated hematopoietic stem cell transplantation for acute myeloid leukemia: results from the multicenter German cooperative study group. *Bone Marrow Transplant* 2005; **35**: 1011–1018.
41 Cao W, Mohacsi P, Shorthouse R, Pratt R, Morris RE. Effects of rapamycin on growth factor-stimulated vascular smooth muscle cell DNA synthesis. Inhibition of basic fibroblast growth factor and platelet-derived growth factor action and antagonism of rapamycin by FK506. *Transplantation* 1995; **59**: 390–395.
42 Haririan A, Morawski K, West MS, El-Amm JM, Doshi MD, Cincotta E *et al*. Sirolimus exposure during the early post-transplant period reduces the risk of CMV infection relative to tacrolimus in renal allograft recipients. *Clin Transplant* 2007; **21**: 466–471.
43 Hill JA, Hummel M, Starling RC, Kobashigawa JA, Perrone SV, Arizón JM *et al*. A lower incidence of cytomegalovirus infection in *de novo* heart transplant recipients randomized to everolimus. *Transplantation* 2007; **84**: 1436–1442.